Remarks

The present invention provides for a method of formulating an insulin composition within a phosphatidylcholine carrier, wherein said insulin is stabilized at room temperature.

Claim Rejections 35 USC § 112

The Examiner has rejected claims 1-6, 8 and 11-16 under 35 USC § 112, first paragraph as failing to comply with the written description requirement. Specifically, the claim limitation "non-liposome multilamellar crystal non-polar phosphatidylcholine" is objected to on the grounds that the topical delivery compositions are non-polar in [0010] but not that the phosphatidylcholine is non-polar and that the topical drug delivery compositions may be in liquid crystal phase but not crystal phase. This rejection is respectfully traversed.

The language of claim 1 recites: "a non-liposomal multilamellar liquid crystal phosphatidylcholine non-polar carrier." The amended claim language does not state "non-polar phosphatidylcholine" but instead recites a "non-polar carrier." The amended claim language is consistent with the indication of the Examiner in the Final Office Action at p. 2 that "the topical delivery compositions are non-polar" and that "the topical drug delivery compositions may be in liquid crystal phase."

Specific support for the claim language is found in the application at ¶[0009], which specifies that the "topical delivery compositions are...nonpolar." The application further specifies at ¶[0013] that:

it is believed that the PPC-enriched phosphatidylcholine forms a bilayer enveloping the polypeptide or macromolecule to create the topical drug delivery composition, contributing to the stability of the active molecule and enhancing penetration. Further, the stabilized insulin compositions formulated in accordance with the present invention may be in liquid crystal phase, with the PPC-enriched phosphatidylcholine loosely arranged in multilamellar fashion, with the polypeptide or macromolecule being bonded and entrapped within the lipid bilayers formed therein. This forms a loosely arranged, yet stable, PPC-enriched phosphatidylcholine-insulin complex that further increases penetration and delivery of the polypeptide or macromolecule to the dermal vasculature.

Clearly the specification as filed defines subject matter which includes a ***multilamellar liquid crystal*** phosphatidylcholine. The ***multilamellar liquid crystal*** has ***bilayers*** entrapping the compound to be administered. Paragraph [0009] further specifies that the topical delivery compositions are ***nonpolar***. Although the term "non-liposomal" is not specifically referred to in the specification, a person of average skill in the art would know that a ***multilamellar liquid crystal*** is not a liposome. It is supported by the disclosure of the specification that the phosphatidylcholine is a multilamellar liquid crystal.

Claims 1-6, 8 and 11-16 are rejected as being indefinite on the grounds that "non-polar carrier" is indefinite. (Final Office Action at 3). It is stated that it is unclear how the carrier can be non-polar when it contains polar phosphatidylcholine. Claims 2-13 are rejected on the grounds that they are dependent on indefinite base claims.

The applicant respectfully submits that the claims are supported by the specification and are not indefinite. Polar molecules form a variety of structures in aqueous media by self-association, depending on water content and temperature. The addition of small amounts of water to many polar lipids results in the initial formation of a reverse micellar solution. As the water content and/or temperature increase, different mesophases such as lamellar, reversed hexagonal, bi-continuous cubic, and isotropic sponge phase are formed. In particular cubic liquid crystals are transparent, isotropic viscous phases and are physically stable in excess water. See e.g. Esposito, *Lipid-Based Supramolecular Systems for Topical Application: A Preformulatory Study,* AAPS PharmSci 2003; 5 (4) Article 30. (Copy submitted herewith). Such crystal mesophases are the "non polar carriers" as claimed in the invention. There are numerous examples polar molecules forming larger nonpolar superstructures (i. e. non-polar carriers). Examples of such from common experience are soaps, detergents, inks, fabric softeners and ski wax. See also, Hansen, U.S. Patent 5,955,502 at Col. 6, lines 33-60.

In this case, the term "non-polar" describes a multilamellar liquid crystal structure which is stable and not prone to form polar structures such as liposomes, emulsomes, micelles or reverse micelles.

The essence of the rejection is that the claimed subject matter is scientifically impossible and thus is indefinite. This is error.

> "Where an applicant has specifically asserted that an invention has a particular utility, that assertion cannot simply be dismissed by Office personnel as being "wrong," even when there may be reason to believe that the assertion is not en-

> tirely accurate. Rather, Office personnel must determine if the assertion of utility is credible (i.e., whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of evidence and reasoning provided). An assertion is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion.

M.P.E.P. §2107.02. No evidentiary support for the rejection has been presented. This does not give rise to a prima facie case of indefiniteness. See e.g., *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992); M.P.E.P. §2107.02. In contrast, Applicant has relied on Esposito, *Lipid-Based Supramolecular Systems for Topical Application: A Preformulatory Study*, AAPS PharmSci 2003; 5 (4) Article 30 (copy submitted herewith); and Hansen, U.S. Patent 5,955,502. In such circumstances, the rejection under 35 U.S.C. §112, second paragraph should be withdrawn.

It is respectfully submitted that a person of ordinary skill in the art will consider theclaim language, and particularly the term "non-polar carrier" to be supported by the written specification and to be definite, and the rejection under 35 USC §112 should be withdrawn.

Claim rejections 35 USC § 103(a)

Claims 1-6, 8 and 11-16 have been rejected as being obvious under 35 U.S.C. §103(a), and therefore unpatentable, over Amselen et al (US 5662932) or Lynch (US 2002/0153509) in view of Hansen (US 4614730) and Patel (US6294192), and with respect to claims 2-6, 8, 15 and 16, Chaiyawat (U.S. 6538061) and Brieva (U.S. 5985298). This rejection is respectfully traversed.

Amselen specifically discloses and is directed at "emulsomes", which are liposome-like structures which are fundamentally different from the loosely arranged multilamellar liquid crystal structure claimed in this application. According to Amselen: "[e]mulsomes of this invention are distinct from standard oil-in-water emulsions. Due to the high phospholipid content of the current invention, a ***monolayer*** of phospholipid surrounds the lipid core at the aqueous interface thereby stabilizing the emulsion. In addition, one or more bilayers or envelopes of phospholipid molecules are believed to form around the particles in many embodiments. " (Amselen, Col. 13, lines 35-45; also Col. 6, lines 50-57.) The phospholipid monolayer is a ***polar*** structure around the lipid core. (Amselen, Col. 6, line 53.)

A key component of the emulsomes taught by Amselem is the presence of an entirely hydrophobic neutral lipid core. The present invention contains no neutral lipid core. In contrast to the 0.5:1 to 1.5:1 phospholipid/core lipid weight ratio as described by Amselem, the phosphatidylcholine/lipid weight ratio of the present invention is 1:0 *since no core lipid is present*. Furthermore, the lipid core in Amselen is ***not*** *a* phospholipid, nor is it phosphatidycholine. It is a triglyceride. (Amselen, Col 4, line 23-Col. 5, line 60). The Examiner's description of Amselen completely omits any mention of the ***monolayer*** of phospholipid that encapsulates the lipid core. It ignores the fact that the monolayer is a ***polar*** structure. The described monolayer in Amselen is highly material to the examination of this application because it is a polar structure that establishes that the disclosed structure in Amselen is a liposome-like structure, not a multilamellar liquid

crystal structure as defined in the formulation method claims of this application. Amselen in its essence merely discloses a liposome which encapsulates a lipid core, and may possibly have additional external bilayer lipid coatings.

Claim 1 of the present application specifies a method of formulating a topical insulin composition comprising: preparing a non-liposome multilamellar liquid crystal phosphatidylcholine non-polar carrier for topical administration; and mixing an insulin solution into said carrier to entrap said insulin within said carrier, wherein said insulin is stabilized at room temperature.

The claimed invention does not have "lipid core" nor the phospholipid "monolayer" disclosed in Amselen. Amselem discloses a very specific structure which is not the same as the claimed invention.

It is a fundamental requirement of a prior art reference that it be enabling of the invention. *In re Paulsen*, 30 F.3d 1475, 1478-79, 31 USPQ2d 1671, 1673 (Fed Cir. 1994)." *Helifix Ltd. V. Blok-Lok, Ltd.*, 208 F.3d 1339, 54 USPQ2d 1299 (Fed. Cir. 2000). Amselen's disclosure is inadequate in that it does not describe the present invention; nor enable the present invention; nor put it the possession of a person of ordinary skill in the art. The entire focus of the patent is on the creation of emulsome structures having a ***polar phospholipid monolayer***, which is ***not*** the same as formulating a ***non-liposomal multilamellar*** liquid crystal phosphatidylcholine ***non-polar*** carrier with insulin as claimed in the present application.

Amselen discloses a particle having a lipid core surrounded by a *polar phospholipid monolayer* and postulates the existence of additional concentric bilayer shells around the lipid core and associated monolayer. (Amselen, Col. 13, lines 35-45; also Col. 6, lines 50-57.) Amselen discloses a liposome-like structure but does not disclose or suggest a ***non-liposomal multilamellar*** liquid crystal phosphatidylcholine ***non-polar*** carrier which stabilizes a drug ***at room temperature.*** Neither Amselen nor the combination of cited references disclose or suggest the claimed invention. The rejection under 35 U.S.C. §103 should be withdrawn.

The combination of Amselen with either the insulin solutions of Hansen, or the polyethylene glycols of Patel would not allow one to arrive at the carrier described within the present invention. The claims of the present invention are therefore patentable over Amselen in view of Hansen and/or Patel. This is similarly true of the teaching of Amselen in view of Hansen and Patel in view of the silicone components of Chaiyawat and Brieva.

Lynch et al teach of a "cubic liquid crystalline phase precursor comprising (A) an ambiphile capable of forming a cubic liquid crystalline phase, (B) an optional solvent and (C) an additive selected from the group consisting of an anchor, a tether and combinations thereof such that 1.0=A+B+C and 1>A>0, 1>B>0 and 1>C>0. (i.e each of A, B and C must be present in some quantity). The "tether" C is identified as having a charged end and a lipid tail in [0055] to [0059]. The carrier of the present invention requires no similar ingredient as that described as the "tether" in Lynch, which requires its presence. Since no comparable C component is present in the carrier of the present

invention the claims of the present invention are therefore patentable over Lynch in view of Hansen and/or Patel. This is similarly true of the teaching of Lynch in view of Hansen and Patel in view of the silicone components of Chaiyawat and Brieva.

Further, prima facie obviousness of the steps of claims 2 and 5, or 11-14, or 15-16 is not shown by the cited prior art. Since neither Amselen nor Lynch discloses the non-liposomal multilamellar liquid crystal phosphatidylcholine non-polar carrier which stabilizes a drug at room temperature of the invention, there would be no motivations to engage in the "judicious selection and routine optimization" which is asserted as the basis for rejection of the dependent claims. Certainly the specific steps specified in claims 2, 5, 11-14, and 15-16 are not disclosed or suggested by the cited prior art.

The combination of two polyglycols with a phaphatidycjholine carrier specified in claims 2 and 4 is not disclosed or suggested by the cited prior art.

Nor is the specific preferred formulation set forth in claim 5 disclosed or suggested by the cited prior art.

The importance of the present invention is that it provides a way of formulating stable topical insulin compositions that were stable for at least 22 weeks at room temperature, which is 21 weeks longer than insulin standards stored at room temperature. [Specification, ¶21]. This is a significant benefit to diabetic patients, particularly those located in geographic regions such as Africa or Asia where reliable refrigeration may not be available. There is no disclosure in Amselen or Lynch or the other art of record of a providing a room temperature stabilized insulin composition as described in the present claims and specification. Neither the disclosed invention nor its

benefits are disclosed or suggested by the combination of references. It is submitted that the presently claimed invention is patentable over Lynch et al. and over Amselem et al. in view of Patel and/or Hansen and Chaiyawat and/or Brieva, and issuance of a Notice of Allowance is respectfully requested.

Respectfully submitted,

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AAPS PharmSci 2003; 5 (4) Article 30 (http://www.aapspharmsci.org).

Lipid-Based Supramolecular Systems for Topical Application: A Preformulatory Study

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ABSTRACT

This article describes the production and characterization of monoglyceride-based supramolecular systems by a simple processing technique, avoiding time-consuming procedures, high energy input, and the use of organic solvents. A preformulatory study was performed to study the influence of the experimental parameters on the production of monoglyceride-based disperse systems. In particular the effects of (1) stirring speed, (2) type and concentration of monoglyceride mixture, and (3) type and concentration of surfactant were investigated on the recovery, fraction of larger particles, mean diameter, and shape of smaller particles (so called nanosomes). Dispersions were first characterized by optical microscopy and freeze-fracture electron microscopy. The mean diameter of standard nanosomes, analyzed by photon correlation spectroscopy (PCS) after elimination of larger particles by filtration, was 193.5 nm. Cryotransmission electron microscopy studies, conducted in order to investigate the structure of dispersions, showed the coexistence of vesicles and particles characterized by a cubic organization. X-ray diffraction data revealed the coexistence of 2 different cubic phases, the first being a bicontinuous cubic phase of spatial symmetry Im3m (Q^{229}) and the second belonging to the Pn3m spatial symmetry. A study on the stability of monoglyceride-based dispersions based on macroscopical analysis of organoleptic properties and dimensional analysis by time was performed after elimination of larger particles by filtration. Organoleptic and morphological features do not change by time, appearing free from phase-separation phenomena for almost 1 year from production. PCS studies showed that nanosomes undergo an initial increase in mean diameter within the first month following production; afterwards they generally maintain their dimensions for the next 4 months.

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INTRODUCTION

Fatty acids and monoglycerides possess antiviral and antibacterial activities.[1-3] It has been demonstrated that enveloped viruses, such as herpes simplex virus type 1 (HSV-1), vesicular stomatitis virus (VSV), and visna virus are inactivated by long-chain unsaturated and medium-chain saturated fatty acids. In particular electron microscopy studies have shown that fatty acids are able to disrupt the lipid envelope of VSV by an unknown mechanism.[1]

Kristmundsdottir et al[4] have studied the activity of a number of medium-chain saturated and long-chain unsaturated fatty acids and their monoglycerides against HSV-1 in order to develop microbicidal hydrogels containing monoglyceride as the active ingredient. In this view, fatty acids and monoglyceride-based formulations can be proposed for intravaginal use as microbicides against sexually transmitted diseases.[5]

Unsaturated long-chain monoglycerides such as monoolein are able to form a variety of structures in aqueous media by self-association, depending on water content and temperature. The addition of small amounts of water to the lipids at 37°C results in the initial formation of a reverse micellar solution. As the water content and/or temperature increase, different

mesophases such as lamellar, reversed hexagonal, bicontinuous cubic, and isotropic sponge phase are formed.[6] In particular cubic liquid crystals are transparent, isotropic viscous phases and are physically stable in excess water.[7-10] Cubic phase represents a unique system for the production of pharmaceutical dosage forms.[11]

Aqueous dispersions of cubic lipid phases can be used for the development of nanoparticulate drug delivery systems characterized by high biocompatibility, bioadhesivity, and easy production protocol.[12] Because of their properties, these versatile delivery systems can be administered orally, parenterally, or percutaneously.[13-15]

Landh and Larsson have patented the preparation of colloidal dispersions of nonlamellar lyotropic crystalline phases and have termed the particles "cubosomes."[16] Cubosomes usually have been produced by means of time-consuming methods involving high energy input. For instance, Gustafsson et al investigated the production and structure of aqueous dispersions of lipid-based lyotropic liquid crystalline phases.[17] The dispersions were based on glycerylmonooleate plus a nonionic triblock polymer (Poloxamer 407) in water. Dispersions were produced by dropwise addition of a melt of lipids and poloxamer in water, followed by reduction of size by homogenization under high pressures at 80°C. Siekmann et al[18] recently reported the preparation and characterization of dispersions composed of monoolein-rich monoglycerides with or without purified soya phospholipids. Dispersions were prepared by equilibration of the monoglyceride/phospholipid/water cubic phase, subsequent fragmentation by a solution of Poloxamer 407, predispersing by probe sonication, and finally high-pressure homogenization. Moreover, some authors have developed experimental protocols for cubosome production based on the use of organic solvents. In particular Spicer and Hayden[19] have proposed a method based on a dilution process of an ethanolic solution of monoolein with an aqueous solution of Poloxamer. Ethanol was used as a hydrotrope to create a liquid precursor, spontaneously forming cubosomes after dilution. Finally Nakano et al[20] have suggested a method for the production of cubosomes based on hydration of a dry film of monoolein/poloxamer with an aqueous buffer. The authors proposed to mix monoolein and poloxamer in chloroform and to dry the mixture by solvent evaporation. After hydration, cubosomes were formed by homogenization at 80°C; the structure of cubosomes was investigated by small-angle x-ray scattering and ^{13}C nuclear magnetic resonance (NMR).

This study examines the production of monoglyceride-based dispersions by a simple processing technique, avoiding time-consuming procedures, multiple equilibration steps, intermediate formation of viscous bulk cubic gel, high energy input, and use of organic solvents.

Aim of this study is to investigate the influence of some experimental parameters on the morphological and dimensional characteristics of monoglyceride-based dispersions to be eventually proposed for topical administration on genital mucosa.

In particular, this study describes (1) a preformulatory study for the production of monoglyceride-based dispersions, (2) the characterization of the dispersions by optical and freeze-fracture electron microscopy, (3) the dimensional analysis by photon correlation spectroscopy (PCS), (4) the characterization of the internal structure of particles by cryotransmission electron microscopy and x-ray diffraction, and (5) an examination of dispersion stability.

MATERIALS AND METHODS

Materials

The monoglyceride mixtures Myverol 18-99 and Myverol 18-92, with fatty acids composition shown in **Table 1**, were kindly donated by Quest International (Lindtsedijk, Holand). The glyceryl monooleate RYLO MG19 (**Table 1**) was a gift from Danisco Cultor (Grindsted, Denmark).

Poloxamer 407 ($PEO_{98}POP_{67}PEO_{98}$) was obtained from BASF (Ludwigshafen, Germany). Celvol 205 (88% hydrolized polyvinylalcohol [PVA]) was from Celanese Chemicals Europe GmbH (Kronberg, Germany). All other materials were of the highest purity grade and were purchased from Fluka Chemicals (Buchs, Switzerland).

Methods

Production of Dispersions

Production of dispersions was based on the emulsification of monoglycerides/surfactant mixtures in water. In particular, the monoglyceride-based lipidic phase was alternatively composed of Myverol 18-99, Myverol 18-92, or RYLO MG19. In addition, mixtures of Myverol 18-99/18-92 (75:25, 50:50, or 25:75 wt/wt) were used.

Poloxamer 407 was used as surfactant in a concentration range between 0% and 20% wt/wt with respect to the disperse phase. The concentration of the monoglyc-

Table 1. Fatty Acid Composition of the Monoglyceride Mixtures as Indicated by Manufacturers*

Fatty Acid	Myverol 18-99	Myverol 18-92	RYLO MG19
Palmitic $C_{16:0}$	4.1	7.0	0.5
Stearic $C_{18:0}$	1.8	4.5	2.2
Oleic $C_{18:1}$	60.9	18.7	92.5
Linoleic $C_{18:2}$	21.0	67.5	4.6
Linoleic $C_{18:3}$	8.8	trace	trace
Gadoleic $C_{20:1}$	1.0	trace	trace
Arachidonic $C_{20:4}$	trace	trace	0.2

*Fatty acid composition given as % wt/wt.

eride/surfactant mixture was between 2.5% and 10% wt/wt with respect to the total weight of the dispersion. Moreover, in some cases PVA was used in addition to poloxamer as a stabilizing agent of the dispersion, by previous solubilization at 80°C in the aqueous phase (1%, 2.5%, or 5% wt/wt).

Typically 2.25 g Myverol 18-99 plus 0.25 g Poloxamer 407 were melted in a water bath (Haake FS bath, Enco sas, Karlsruhe, Germany).

The obtained molten mixture was added dropwise into 47.5 mL of water at 70°C under mechanical stirring (Eurostar digital stirrer, IKA Labotechnik, Sardo, Torino, Italy) at different speeds (ie, 500, 750, 1000, 1250, and 1500 rpm).

Dispersions were maintained under stirring and were cooled to room temperature up to the solidification of lipid droplets (after 2 hours). In some cases, cooling was conducted using an external ice bath for 10 to 15 min.

Alternatively the dispersions were subjected to sonication using a bath sonicator (Branson 2200, Branson Ultrasonic, Danbury, Connecticut) for 1 minute or to homogenization at 5000 rpm (Ultra Turrax, Janke & Kunkel, Ika-Werk, Sardo, Italy) for 1 minute. After cooling, the dispersions were observed by optical microscopy.

The disperse phase that was lost on the paddle of the overhead mechanical stirrer was recovered and weighed. The dispersions were then weighed in order to evaluate the water evaporation due to high temperature and rapid stirring during production. The extent of water loss due to evaporation was calculated as shown in Equation 1:

$$\text{water loss} = W\,MO/P407/H_2O\_(W dp + W disp) \quad (1)$$

where W *MO/P407/H_2O* is the weight of monoglyceride/poloxamer and water before dispersion; *Wdp* is the weight of disperse phase lost on the paddle; and *Wdisp* is the weight of dispersion after production. The dispersion was then filtered through mixed esters of cellulose membrane (0.6 µm pore size) in order to separate big monoglyceride-poloxamer aggregates. After filtration, both dispersion and filter were weighed. Finally, the filter was left to desiccate in an oven at 70°C for 12 hours and again weighed. Dispersions were stored in glass vials at 25°C.

Characterization of Dispersions

Monoglyceride-based dispersions were characterized by optical microscopy, freeze fracture and cryotransmission electron microscopy. Optical analyses were performed by an optical inverted microscope (Nikon Diaphot, Nikon, Tokyo, Japan) equipped with a Nikon F-601 camera. After production, 500 µL of dispersion was placed in a microscopy glass, over the objective of the microscope, and left to equilibrate. Photomicrographs were taken only after 40 to 60 seconds in order to let small nanosomes slow down their visible movements. Magnification was between ×250 and ×1000.

Particle morphology was characterized by freeze-fracture electron microscopy. Samples of dispersions were frozen by the "propane jet" technique, cryofixed, and microfractured at 108°K (Balzers BAF 300, Baltec., Balzers, Lichtenstein, Germany) with a 10^{-5} Pa pressure; replicas were reproduced by a platinum/carbone matrix. For the electron microscopy analysis, a Philips EM 301 (FEI, MEindhoven, The Netherlands) was employed at 100 kV. Photographs were taken on Agfa Scientia 23D56 film (Science Services, Muenchen, Germany) and developed by a MohrPro (Science Services, Muenchen, Germany) for 3.5 minutes at 293°K.

For cryotransmission electron microscopy studies, a drop of the sample was put on an untreated pure copper transmission electron microscopy (TEM) grid (600 mesh, Science Services, Muenchen, Germany), where most of the liquid was removed with blotting paper leaving a thin film stretched over the grid holes. The specimens were instantly shock frozen by rapid immersion into liquid ethane and cooled to approximately 90K by liquid nitrogen in a temperature-controlled freezing unit (Zeiss Cryobox, Oberkochen, Germany). The temperature was monitored and kept constant in the chamber during all the sample preparation steps. After freezing the specimens, the remaining ethane was removed using blotting paper. The specimen was inserted into a cryotransfer holder and transferred to a Zeiss CEM902A TEM (LEO, Oberkochen, Germany), equipped with a cryostage. Examinations were carried out at temperatures between 77K and 80K. The TEM was operated at an acceleration voltage of 80 kV and a beam current of approximately 1 μA. A condenser diaphragm of 100 μm and an objective entrance aperture of 17 mrad corresponding to a diaphragm diameter of 90 μm were used for imaging. Zero-loss filtered images were taken under low-dose conditions (ie, using the minimal dose focusing device). The applied radiation dose was in the range of 0.1 Coulomb (C) / cm^2 at a primary magnification of ×30 000. All images were registered digitally by a low-dose TV rate camera system (Dage, SIT 66, MTI, Michigan City, IN) combined and processed with a digital imaging processing system.

Submicron particle size analysis was performed on filtered dispersions using a Zetasizer 3000 PCS (Malvern Instruments, Malvern, UK) equipped with a 5-mW helium neon laser with a wavelength output of 633 nm. Glassware was cleaned of dust by washing with detergent and rinsing twice with water for injections. Measurements were made at 25°C at an angle of 90 degrees with a run time of at least 180 seconds. Data were interpreted using the method of cumulants.

X-Ray Diffraction Measurements

Diffraction measurements were carried out using a Philips PW 1830 x-ray generator (Philips) equipped with a Guinier-type focusing camera (Philips, Eindhoven, The Netherlands) operating in vacuum with a bent quartz crystal monochromator, which selects for the K_α line ($\lambda = 0.154$ nm). Diffraction patterns were recorded on a stack of 3 Kodak DEF-392 films or using an Inel CPS (curved position sensitive) 120 detector (Inel, Artenay, France). Glass capillaries with a diameter of 3.0 mm and a wall thickness of 0.01 mm, filled with the monoglyceride samples, were used for the x-ray diffraction measurements.

Diffraction data were collected at 25 °C and 37 °C, controlling the temperature with a Haake F3 thermostat (ThermoHaake, Karlsruhe, Germany) with an accuracy of 0.1°C. Before each measurement, the sample was maintained in equilibrium at the desired temperature for 10 minutes.

Stability Studies

In order to assess the physical stability of monoglyceride dispersions, some organoleptic and morphological aspects such as odor, color, phase separation, and formation of precipitates were investigated as a function of time. In addition, PCS studies were repeated at different time intervals to evaluate possible variations of particles' dimensional distribution.

RESULTS AND DISCUSSION

Production of Monoglyceride-Based Dispersions

The emulsification of monoglyceride/surfactant mixtures in water results in formation of supramolecular systems constituted of lipidic particles and vesicles in the nanometer dimensional range. In this study, dispersion production was first performed by a disperse phase composed of Myverol 18-99 and Poloxamer 407 90:10 wt/wt. The dispersing phase, composed of water, was 95% with respect to total weight of the dispersion. In contrast to other methods described in the literature, which usually rely on time-consuming and high energy input techniques[12,16-18] or on the use of organic solvents,[19-20] in the present study more conventional dispersion techniques were tested. In particular (1) different experimental protocols based on the use of a sonicator bath or an overhead mechanical stirrer, (2) different cooling modalities, and (3) the homogenization of the dispersion after production were investigated.

The use of sonication resulted in the instantaneous production of a big disperse phase aggregate, preventing emulsion formation, whereas the overhead mechanical stirrer achieved milky dispersions with few macroscopic aggregates (as evidenced by visual inspection).

Regarding cooling modalities, it was found that a rapid cooling of the emulsion using an external ice bath led to formation of a number of big monoglyceride aggregates; conversely a slow cooling at room temperature enabled formation of milky dispersions with few macroscopic aggregates. Moreover, the possibility was investigated of subjecting the dispersion to homogeniza-

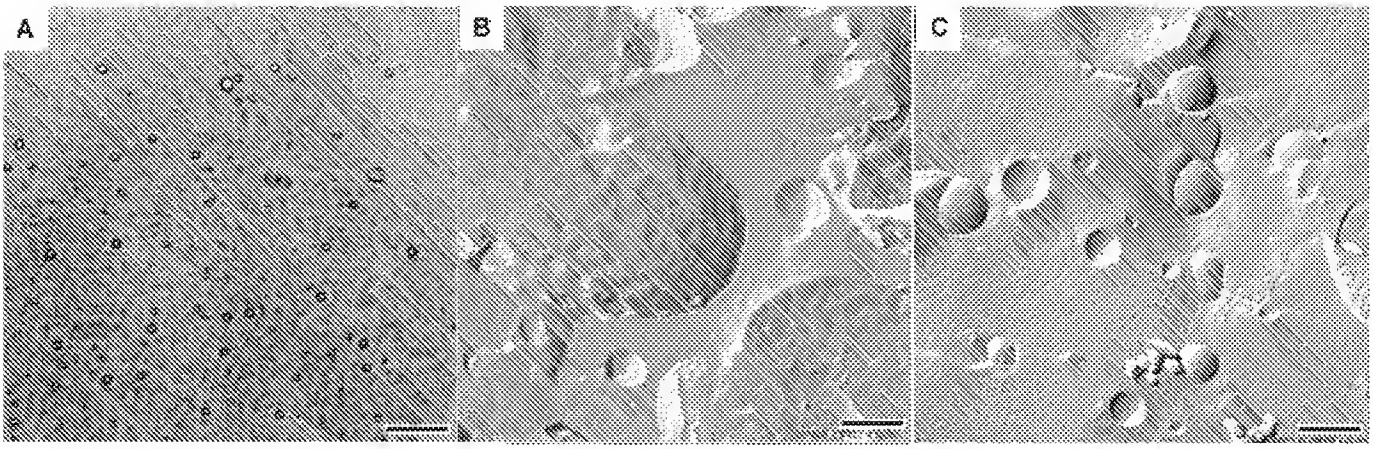

Figure 1. Optical (A) and freeze-fracture electron (B, C) micrographs of a monoglyceride-poloxamer-based dispersion. The disperse phase was composed of Myverol 18-99/Poloxamer 407 90:10 wt/wt; the disperse phase/dispersing phase ratio was 5: 95; the dispersion was produced by an overhead mechanical stirrer with a 1500 rpm stirring speed. The bar equals 30 μm in (A), 400 nm and 200 nm in (B) and (C), respectively.

tion immediately after production, in order to possibly reduce the number of aggregates, nevertheless this step increased aggregate formation.

Thus the best experimental conditions for the production of dispersions were emulsification of monoglyceride-poloxamer in water by an overhead mechanical stirrer followed by slow cooling to room temperature. **Figure 1** shows photomicrographs of a typical dispersion (produced with the above reported composition with a 1500 rpm stirring speed) taken by optical (A) or freeze-fracture electron (B, C) microscopy. Optical microscopy observation (Panel A) provides a general overview of the monoglyceride dispersion, evidencing the presence of at least 2 particle populations of different sizes.

From electron microscopy images, the heterogeneous morphology of particles in the same dispersion can be observed. In particular, Panel B shows the irregular shape of larger particles, while Panel C evidences the regular morphology of the smaller particles (nanosomes) characterized by a spheroidal shape.

After production, recovery of dispersions was calculated taking into account the loss of disperse phase on the paddle of the overhead mechanical stirrer and the loss of dispersing phase due to water evaporation (Equation 1). To this aim both the disperse phase lost on the paddle and the dispersion were weighed. In particular, the loss of disperse phase and the weight of dispersion were found to be 0.5% ± 0.02% and 88% ± 0.01%, respectively, with respect to water-monoglyceride-poloxamer weight before production (ie, the whole dispersion). Thus the extent of water loss after stirring at 1500 rpm (calculated by difference) was about 11.5%, even though it should be considered that a certain amount of water was taken up by the lost disperse phase. In particular, to provide a more realistic view of the situation, a rough model calculation of the real water loss of a standard dispersion is provided in Equation 2.

$$\text{water loss} = 50\text{ g} - (\text{W}\,MO/P407/H_2O) - (0.25\text{ g} + 44\text{ g})(\text{W}dp + \text{W}disp) = 5.75\text{ g} \quad (2)$$

where W $MO/P407/H_2O$ is the weight of monoglyceride/poloxamer and water before dispersion; *Wdp* is the weight of disperse phase lost on the paddle; and *Wdisp* is the weight of dispersion after production.

Recovery data were the mean of 8 different batches of the same type of dispersion. With the aim to separate the larger particles from the smaller, dispersions were filtered through mixed esters of cellulose membrane (0.6 μm pore size). The filter was then left to desiccate in order to eliminate as much as possible of the water taken up by the particles. The weight of the larger particles after desiccation was 28% ± 0.5% with respect to monoglyceride-poloxamer weight before production (data were the mean of 8 different batches of the same type of dispersion). It was found that the uptaking of water by large particles was 5.6-fold with respect to their weight, namely 25.2% wt/wt (taking into account the 4.5% wt/wt concentration of monoglycerides). This result is not in complete agreement with the literature data, which report that a sample of monoolein put in excess water can form the cubic phase by swelling to a higher water content (~35% wt/wt).[7,9]

Table 2. Effect of the Stirring Speed on the Characteristics of Dispersions*

Stirring Speed (rpm)	Recovery† *Water Loss*‡ (% wt/wt)§	Lost on the Paddle ‖ (% wt/wt)§	Large Particles (%)¶	Mean Diameter Z Average (nm)#	PI
500	89.0 *8.9*	2.1	30.1 ± 0.6	512.5	0.03
750	87.3 *9.5*	0.2	30.2 ± 0.7	509.1	0.05
1000	86.2 *10.5*	3.3	30.3 ± 0.4	460.9	0.14
1250	85.6 *11*	3.4	30.1 ± 0.8	349.8	0.09
1500	88.0 *11.5*	0.5	28.0 ± 0.5	193.5	0.18

*PI indicates polydispersity index. PCS data were the mean of 5 determinations on different batches of the same type of dispersion. SD was between ± 0.5 for mean diameter values and between ± 5% for PI values. Dispersions were produced by a disperse phase composed of Myverol 18-99/Poloxamer 407 90:10 wt/wt in water with a 5:95 disperse phase/dispersing phase ratio.
†Weight of dispersion after production
‡Weight loss due to water evaporation
§With respect to the total weight of the material employed for the dispersion
‖Loss of disperse phase on the paddle of the mechanical overhead stirrer
¶Fraction of large particles after filtration of dispersion, with respect to the weight of the disperse phase before dispersion production; data were the mean of 8 different batches of the same type of dispersion
#Determined by PCS

It should be underlined that the size distribution of the dispersion was analyzed by PCS after filtration, without taking into account the fraction of larger particles whose dimensions ranged from 0.9 to 30 μm, as measured by electron microscopy observations. Nanosomes were characterized by a monomodal dimensional distribution and an intensity mean diameter of 193.5 nm, expressed as Z Average (**Table 2**).

In order to study the influence of the experimental parameters on the production of monoglyceride-based disperse systems, a preformulatory study was conducted starting from the above reported composition and concentration of disperse and dispersing phases. In particular the effects of (1) mechanical stirring speed, (2) type and concentration of monoglyceride mixture, and (3) type and concentration of surfactant were investigated on recovery, fraction of larger particles, shape and mean diameter of nanosomes.

Effect of Stirring Speed

As reported above, the best stirring system for production of dispersions composed of Myverol 18-99 and Poloxamer 407 (90:10 wt/wt) in water (95% with respect to the total weight of dispersion) was an overhead mechanical stirrer. The stirring speed used in the emulsification step was first fixed at 1000 rpm, resulting in the formation of many aggregated particles, as observed by optical microscopy (data not shown). Freeze-fracture electron micrograph evidenced the irregular shape of particles (**Figure 2A**). Filtration achieved the separation of the larger particles (representing 30% wt/wt with respect to monoglyceride-poloxamer weight before production) from the smaller nanosomes, having a mean diameter of 460.9 nm. Second, stirring speed was lowered to 500 or 750 rpm and raised to 1250 rpm without changes in the fraction of larger particles. **Figure 2B** shows a freeze-fracture electron image of a dispersion produced at 500 rpm, evidencing the presence of 2 particle populations of different sizes. In contrast, the use of a 1500 rpm stirring speed resulted in the formation of a minor fraction of larger particles (28% wt/wt) and nanosomes characterized by spheroidal shape (**Figure 1C**, **Table 2**). Mean diameters of nanosomes were between 193.5 (1500 rpm stirring speed) and 512.5 nm (500 rpm stirring speed), the faster the stirring speed, the lower the mean diameter. This result is in agreement with the Arshady equation, which relates particle mean diameter with some important experimental parameters such as the stirring speed.[21] Percentages of recovery were always greater than 85% wt/wt. The amount of disperse phase lost on the paddle was very high (~2%-3% wt/wt) at lower

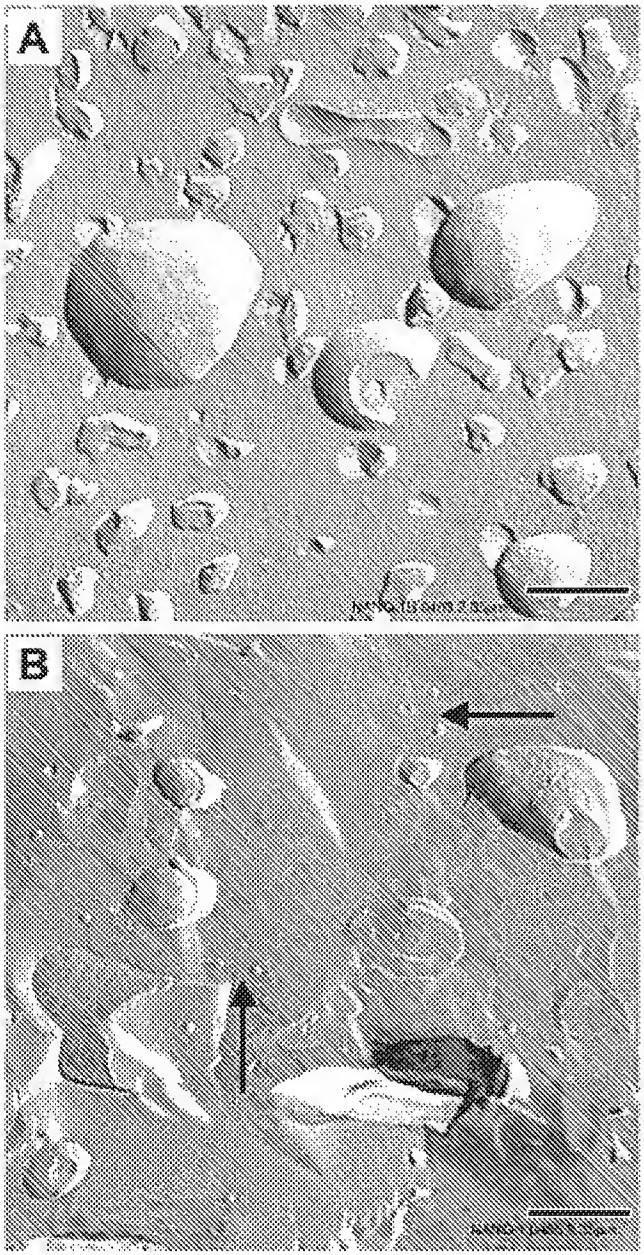

Figure 2. Freeze-fracture electron micrographs of dispersions produced by an overhead mechanical stirrer with a 1000 rpm (A) or 500 rpm (B) stirring speed. The disperse phase was constituted of Myverol 18-99/ Poloxamer 407 90:10 wt/wt, the disperse phase/ dispersing phase ratio was 5: 95. The arrows in (B) indicate some particles of the smaller size range. The bar equals 860 nm and 2.5 μm in (A) and (B), respectively.

stirring speeds, while it was just 0.5% wt/wt when the highest stirring speed was used. As expected, weight losses due to water evaporation were a function of the stirring speed: the higher the stirring speed, the higher the water loss (**Table 2**). All further experiments were continued with a stirring speed of 1500 rpm.

Effect of Type and Concentration of Monoglyceride

First, the production of dispersions was based on the emulsification of the molten mixture Myverol 18-99/Poloxamer 407 (90:10 wt/wt) in water. The use of a 5% concentration of disperse phase (with respect to the total weight of dispersion) led to formation of nanosomes characterized by a spheroidal shape, a mean diameter of 193.5 nm, and a monomodal dimensional distribution (**Table 3**, **Figure 1**). In these conditions the fraction of larger particles represented 28% wt/wt with respect to the weight of the disperse phase before production.

In order to study the effect of the concentration of disperse phase on the formation of dispersions, the concentration of the monoglyceride/surfactant mixture was alternatively lowered to 2.5% or raised to 10% wt/wt. The lower disperse phase concentration resulted in the greatest amount lost on the paddle (4.1% wt/wt with respect to the weight of dispersion before production) in a high fraction of large particles (32.1% wt/wt with respect to the weight of disperse phase) and in formation of nanosomes with a mean diameter of 154 nm (**Table 3**). The higher disperse phase concentration led to formation of a 29% wt/wt fraction of large particles and smaller nanosomes characterized by a bimodal dimensional distribution and a mean diameter of 241 nm. The freeze-fracture electron micrograph in **Figure 3A** shows few nanosomes produced by the use of 2.5% wt/wt disperse phase, while the optical micrograph in **Figure 3B** shows many big aggregates obtained by the use of 10% wt/wt disperse phase. Percentages of recovery were between 85.2% and 88% wt/wt (**Table 3**). A further increase in the concentration of disperse phase to 12% wt/wt did not lead to nanosome formation but only resulted in a number of aggregates.

Second, the possibility of replacing Myverol 18-99 with increasing amounts of Myverol 18-92 (composition reported in **Table 1**) was investigated. It was found that both the amount of disperse phase lost on the paddle and the fraction of larger particles increased with the use of increasing concentrations of Myverol 18-92, up to the complete absence of nanosomes in the case of the use of pure Myverol 18-92 (**Table 4**). This surprising result could be related to the different composition of the monoglyceride mixture employed; experiments are in progress in order to investigate this matter.

Table 3. Effect of Different Concentrations of Phase Disperse on the Characteristics of Dispersions*

Phase Disperse Concentration (% wt/wt)	Recovery† *Water Loss*‡ (% wt/wt)§	Lost on the Paddle‖ (% wt/wt)§	Large Particles (%)¶	Mean Diameter Z Average (nm)#	PI
2.5	84.42 *11.5*	4.1	32.1 ± 0.6	154.0	0.24
5	88.0 *11.5*	0.5	28.0 ± 0.5	193.5	0.18
10	86.34 *11.6*	2.1	29.1 ± 0.4	241.0	0.25

*Dispersions were produced by a disperse phase composed of Myverol 18-99/Poloxamer 407 90:10 wt/wt in water with a 1500 rpm stirring speed. PI indicates polydispersity index. Additional footnotes are as explained in Table 2.

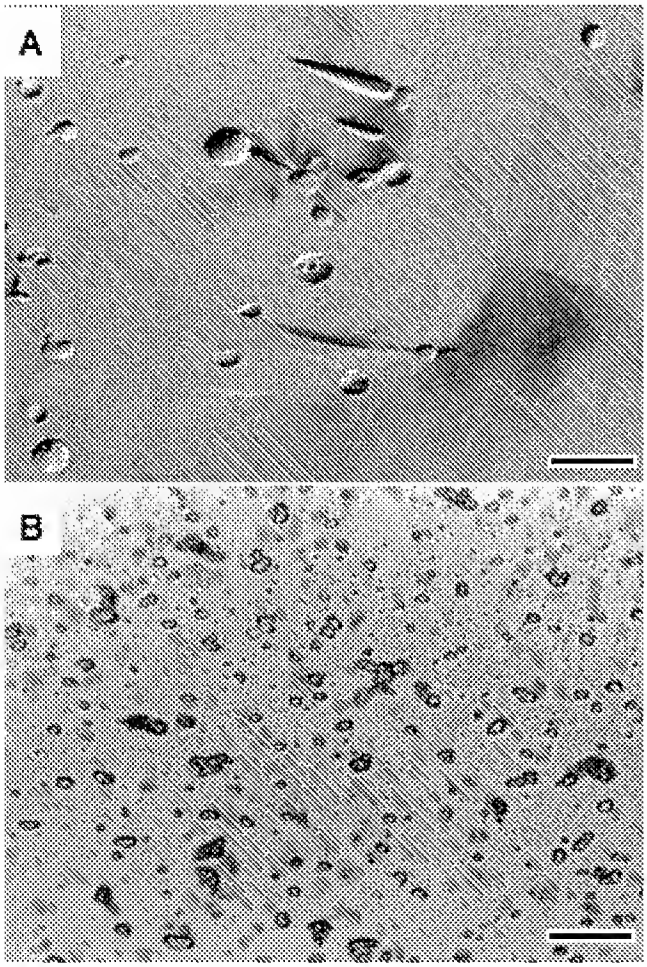

Figure 3. Freeze-fracture electron and optical micrographs of monoglyceride dispersions. The concentration of disperse phase was (A) 2.5% or (B) 10% wt/wt. The disperse phase was composed of Myverol 18-99/Poloxamer 407 90:10 wt/wt; dispersions were produced by an overhead mechanical stirrer with a 1500 rpm stirring speed. The bar equals 460 nm and 25 μm in (A) and (B), respectively.

Mean diameter of nanosomes was affected by the proportion of monoglyceride used: the higher the fraction of Myverol 18-92, the higher the nanosome diameter (ranging from 226.3 nm in the case of Myverol 18-92 [25% wt/wt] to 447.3 nm in the case of Myverol 18-92 [75% wt/wt] [**Table 4**]).

Figure 4 shows optical (Panel A) and electron (Panel B) micrographs of a RYLO MG19/ poloxamer-based dispersion. The use of RYLO MG19 resulted in a 29% wt/wt fraction of large particles, a 1.6% wt/wt loss of disperse phase on the paddle, as well as the formation of nanosomes characterized by a mean diameter of 198.5 nm and a spherical shape, as evidenced by electron microscopy analysis (**Figure 4B**).

Effect of Type and Concentration of Surfactant

It is well known that glycerolmonoleate itself does not form a stable emulsion in water, requiring an emulsifier.[18,22,23] As reported by many authors, poloxamer was found to drastically increase the stability of the vesicle state occurring in lipid dispersions.[17,20,22,23] In particular, Poloxamer 407 was demonstrated to efficiently stabilize dispersions of hexagonal and bicontinuous cubic phases.[17] As reported elsewhere, the phase diagram of monoolein/Poloxamer 407 evidences that the surfactant is not merely absorbed at the particle surface.[20] In particular, it is suggested that the polypropylene oxide (PPO) blocks of Poloxamer are anchored in the apolar region or at the surface of the monoglyceride-based bilayers, while the polyethylene oxide (PEO) tails are solubilized in the water.[23] This disposition should stabilize the vesicles toward fusion by a strong steric repulsion between bilayers.

In this study, the stabilization of monoglyceride dispersions was performed by the addition of different amounts of poloxamer to the disperse phase.

Table 4. Effect of Different Concentrations of Monoglycerides on the Characteristics of Dispersions*

Glycerides	Recovery† *water loss*‡ (% wt/wt)§	Lost on the Paddle ‖ (% wt/wt)§	Large Particles (%)	Mean Diameter Z Average (nm)#	PI
Myverol 18-99	88.0 *11.5*	0.5	28 ± 0.5	193.5	0.18
Myv 18-99/18-92 (75 : 25 wt/wt)	88.0 *11.2*	0.8	33.1 ± 0.2	226.3	0.11
Myv 18-99/18-92 (50 : 50 wt/wt)	87.3 *11.7*	1.0	42.2 ± 0.5	280.3	0.05
Myv 18-99/18-92 (25 : 75 wt/wt)	85.6 *11.5*	2.9	60.3 ± 0.7	447.3	0.12
Myverol 18-92	/ *11.5*	3.8	100 ± 0.0	/	/
RYLO MG19	87.0 *11.4*	1.6	29.1 ± 0.3	198.5	0.10

*Dispersions were produced by a disperse phase composed of monoglycerides/Poloxamer 407 90:10 wt/wt in water with a 5:95 disperse phase/dispersing phase ratio. The stirring speed was 1500 rpm. Myv indicates Myverol; PI, polydispersity index. Additional footnotes are as explained in Table 2.

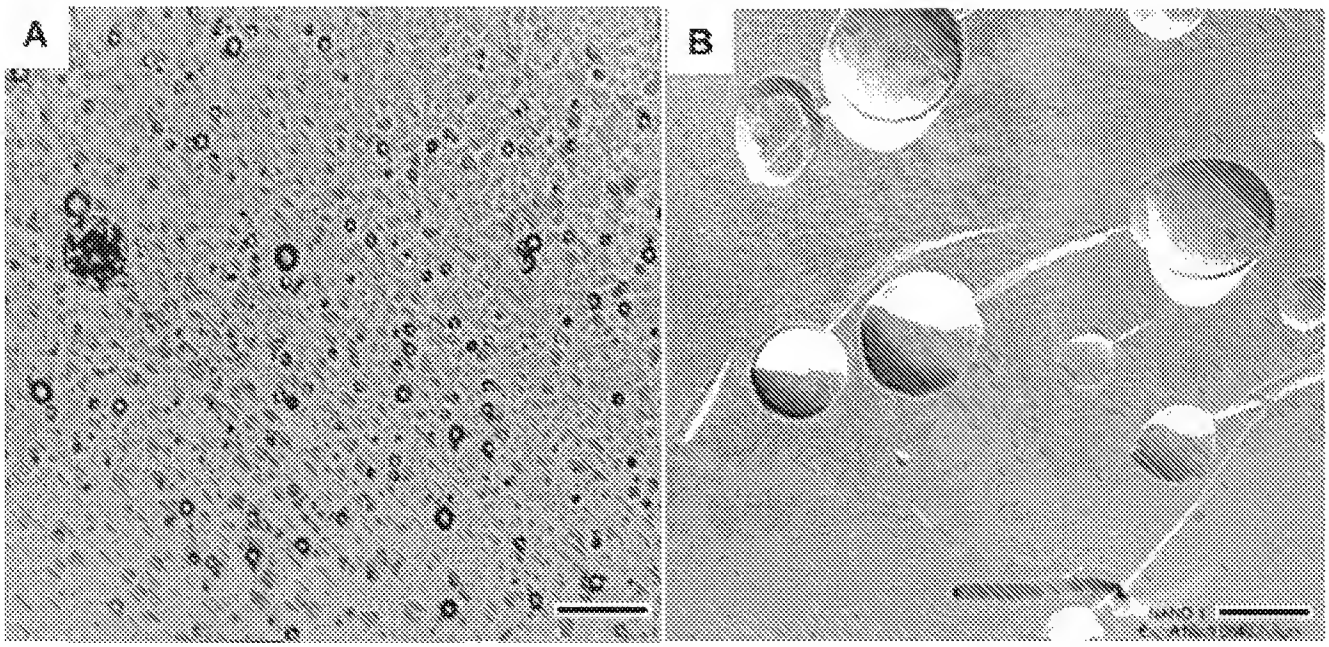

Figure 4. Optical and freeze-fracture electron micrographs of a monoolein-poloxamer-based dispersion. The disperse phase was constituted of RYLO MG19/Poloxamer 407 90:10 wt/wt; the disperse phase/dispersing phase ratio was 5:95; the dispersion was produced by an overhead mechanical stirrer with a 1500 rpm stirring speed. The bar equals 30 μm and 150 nm in (A) and (B), respectively.

Monoglyceride dispersions were produced in the absence of surfactant, resulting in very aggregated particles (52% wt/wt with respect to the weight of disperse phase), a high loss of disperse phase on the paddle (3.5% wt/wt with respect to the weight of dispersion), and a mean diameter of 536.1 nm. The amount of poloxamer was then progressively increased up to 20% wt/wt (with respect to the disperse phase used for

Table 5. Effect of Different Concentrations of Poloxamer on the Characteristics of Dispersions*

Poloxamer 407 (% wt/wt)	Recovery† *Water Loss*‡ (% wt/wt)§	Lost on the Paddle‖ (% wt/wt)§	Large Particles (%)	Mean Diameter Z Average (nm)#	PI
0	85.1 *11.4*	3.5	52.1 ± 0.3	536.1	0.04
2.5	85.1 *11.8*	3.1	40.1 ± 0.1	480.7	0.05
5	88.0 *11.4*	0.6	37.2 ± 0.6	440.5	0.01
7.5	88.3 *11.2*	0.5	34.1 ± 0.7	263.3	0.17
10	88.0 *11.5*	0.5	28.0 ± 0.5	193.5	0.18
15	88.3 *11.5*	0.2	32.1 ± 0.4	214.6	0.13
20	85.5 *11.6*	2.9	38.1 ± 0.5	340.7	0.01

*Dispersions were produced by a disperse phase composed of Myverol 18-99/Poloxamer 407 and water, a 5:95 disperse phase/dispersing phase ratio and a 1500 rpm stirring speed. PI indicates polydispersity index. Additional footnotes are as explained in Table 2.

nanosome production) (**Table 5**). As the amount of poloxamer used increased, up to 10% wt/wt, the fraction of larger particles and the mean diameter of smaller particles decreased, leading to formation of nanosomes with a mean diameter of 193.5 nm and few aggregates (**Figure 1**). The use of higher poloxamer concentrations resulted in both an increase of large particles and an increase in the mean diameter of smaller particles. These results suggest that the concentration of poloxamer employed could affect the steric stability of the dispersions; similar findings were reported by Nakano et al.[20]

Moreover, in the present study, the effect of the presence of PVA in the external aqueous phase was investigated with the aim to study its possible influence on morphology and size of nanosomes. Many authors report the use of PVA as a stabilizing agent in the production of polymeric micro- and nanoparticles[24-27] because PVA is known to act as dispersing agent able to increase the viscosity of the external aqueous phase thereby increasing emulsion stability. PVA is also able to stabilize the emulsion, acting as a protective polymer by being adsorbed at the oil/water interface of droplets during particle formation. In addition, PVA decreases the coalescence of the particles and decreases the particle size.

In particular, in this study, PVA 1%, 2.5%, or 5% wt/wt was solubilized in the aqueous external phase before dispersion production. **Table 6** summarizes the characteristics of the obtained nanosomes, showing that the use of PVA achieved a percentage of larger particles between 27% and 29% wt/wt (with respect to the weight of disperse phase) and spherical nanosomes, with mean diameters between 152 and 247.5 nm. The addition of PVA 1% wt/wt resulted in an increase of the mean diameter of nanosomes (247.5 nm) with respect to the PVA-free standard dispersion (193.5), while an increase in PVA concentration led to the production of nanosomes with lower mean size. In particular, the use of the highest PVA concentration (5% wt/wt) achieved a decrease in mean size to 152 nm, to minimize the aggregate formation and the amount of disperse phase lost on the paddle (0.2% wt/wt, with respect to the weight of dispersion).

The preformulatory study enabled the selection of the so called "standard condition" for the production of nanosome dispersions. In particular, the use of a stirring speed of 1500 rpm, Myverol 18-99 (5% wt/wt) (with respect to weight of dispersion), and Poloxamer 407 (10% wt/wt) (with respect to the disperse phase) enabled the production of dispersions presenting 28% of larger irregular particles and nanosomes characterized by spheroidal shape, few aggregates, mean diameter of 193.5 nm, and high percentage of recovery (88% wt/wt).

In order to shed light on the internal structure of the dispersed particles in monoglyceride-based dispersions produced by the standard conditions, cryo-TEM analy-

Table 6. Effect of Different Concentrations of PVA on the Characteristics of Dispersions*

PVA Concentration (% wt/wt)	Recovery† *Water Loss*‡ (% wt/wt)§	Lost on the Paddle‖ (% wt/wt) §	Large Particles (%)¶	Mean Diameter Z Average (nm)#	PI
1	87.80 *11.5*	0.7	28.5 ± 0.8	247.5	0.17
2.5	88.00 *11.5*	0.5	28.0 ± 0.5	190.0	0.23
5	88.00 *11.8*	0.2	27.1 ± 0.4	152.0	0.18

*Dispersions were produced by a disperse phase constituted of Myverol 18-99/Poloxamer 407 90:10 in water, a 5:95 disperse phase/dispersing phase ratio and a 1500 rpm stirring speed. PI indicates polydispersity index. Additional footnotes are as explained in Table 2.

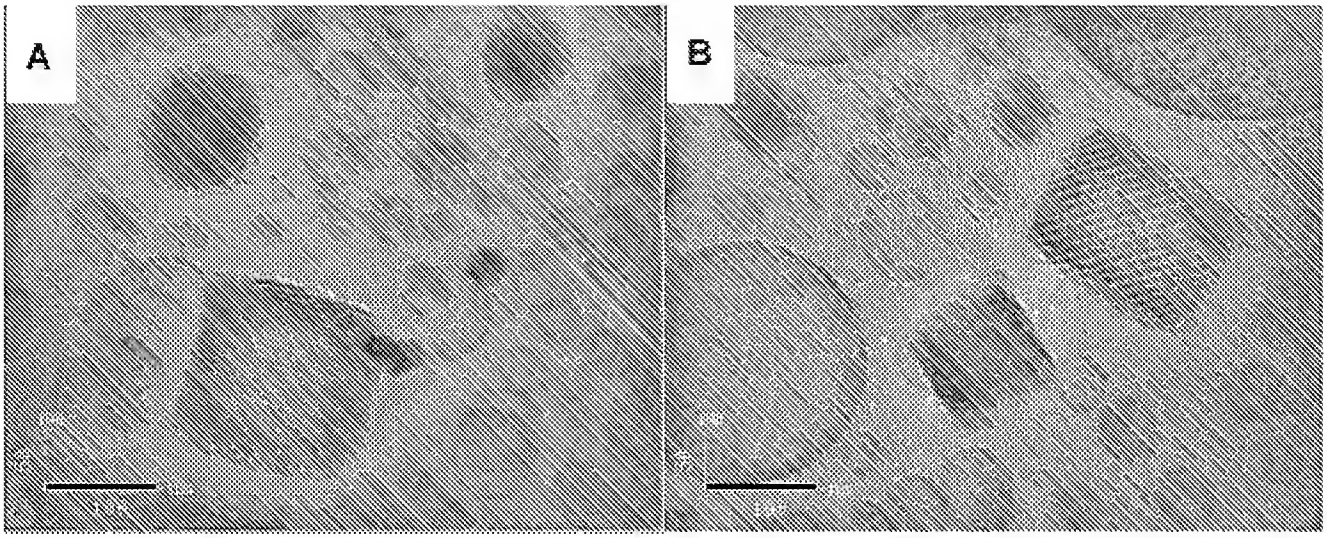

Figure 5. Cryo-TEM micrographs of monoglyceride-based dispersions composed of (A) Myverol 18-99 or (B) RYLO MG19 and Poloxamer 407 90:10 wt/wt. The disperse phase/dispersing phase ratio was 5: 95; dispersions were produced by an overhead mechanical stirrer with a 1500 rpm stirring speed.

ses have been conducted. **Figure 5** shows 2 micrographs, which clearly show the heterogeneous morphology of the disperse phase, both for (Panel A) Myverol 18-99- and (Panel B) RYLO MG19-based dispersions. In particular, the coexistence of spherical vesicles and few faceted particles with well-shaped cubosomes exhibiting the typical ordered cubic texture can be observed. Vesicular structures appear also attached on the surface of cubosomes, as found by other authors, suggesting that over time a transformation may take place from conglomerates of partially fused vesicles to well-ordered particles.[17,22,23]

X-Ray Diffraction Analyses

With the aim to determine the structural organization of different monoglyceride mixtures/Poloxamer 407 in water, x-ray diffraction experiments have been performed both on Myverol 18-99 and RYLO MG19-based dispersions produced by the standard conditions. The experiments have then been carried out at 25°C (room temperature) and 37°C (body temperature) on systems that have not been previously filtered.

Several Bragg peaks were observed in each experiment and their spacing measured. Unambiguous assignment of the lipid mesophases was not possible because of the low number of low-angle reflections observed. This low number of low-angle reflections is the result of the high sample dilution and the presence of a large diffuse low-angle scattering that can be related to the presence of large micelles and/or vesicles. Nevertheless, in all the samples, a series of Bragg peaks, which can be indexed according to the Im3m lattice, Q^{229} (spacing ratios: $\sqrt{2}$: $\sqrt{4}$: $\sqrt{6}$: $\sqrt{8}$: $\sqrt{10}$...), was detected. Other low-

Table 7. X-ray Diffraction Data for Some Monoglyceride/Poloxamer Dispersions*

Dispersion	25°C		37°C	
	Space Group	Unit Cell, *a* (nm)	Space Group	Unit Cell, *a* (nm)
Myverol 18-99/P407	Im3m	15.6 ± 0.2	Im3m	13.5 ± 0.1
	Pn3m	16.2 ± 0.2	Pn3m	15.1 ± 0.2
RYLO MG19/P407	Pn3m	16.8 ± 0.2	Pn3m	16.8 ± 0.2
	Im3m	12.5 ± 0.1	Im3m	12.5 ± 0.1

*Dispersions were produced by a disperse phase composed of monoglycerides/Poloxamer 407 90:10 wt/wt in water with a 5:95 disperse phase/dispersing phase ratio. The stirring speed was 1500 rpm.

angle reflections were also observed. The peak sequence is compatible with the presence of the Pn3m, Q^{224}, bicontinuous cubic phase (spacing ratios: $\sqrt{2}$: $\sqrt{3}$: $\sqrt{4}$: $\sqrt{6}$: $\sqrt{8}$: $\sqrt{9}$...), already detected in analogous systems,[17,18,28,29] even if the presence of a cubic phase of spatial symmetry P4(3)32, Q^{212} (spacing ratios: $\sqrt{2}$: $\sqrt{3}$: $\sqrt{5}$: $\sqrt{6}$: $\sqrt{8}$: $\sqrt{9}$...),[30] cannot be completely excluded. In fact, the Pn3m and the P4(3)32 low-angle diffraction patterns only differ in the position of the third diffraction order peak ($a/\sqrt{4}$ and $a/\sqrt{5}$, respectively, where a is the unit cell dimension (see below)). It should be noted that the P4(3)32 phase is an inverse cubic phase, which has been observed to form by dehydrating monoolein in the presence of proteins and is characterised by a unique double organization: a network of rods embedding a matrix of micelles.[31]

X-ray diffraction results are in agreement with cryo-TEM observations (**Figure 5**), revealing the coexistence of vesicles and particles characterized by a cubic organization. It should be emphasised that it is not possible by cryo-TEM to unambiguously determine the exact nature of the structures formed.[32] Therefore, cryo-TEM cannot help in assigning the Pn3m or the P4(3)32 symmetry to our mixtures. However, according to previous results in these kinds of systems, we suggest that the Pn3m bicontinuous cubic phase is most likely to occur and we will refer to it in our discussion.

Once the symmetry of the lipid phase was found, the unit cell dimension, a, was calculated according to the Bragg's Law as follows:

$$a = (h^2 + k^2 + l^2)^{1/2} / s_{hkl} \qquad (3)$$

where $s_{hkl} = 2 \sin \vartheta / \lambda$ with 2ϑ being the scattering angle and λ (0.154 nm), the wavelength.

X-ray diffraction data are reported in **Table 7**; the spatial symmetry detected for each dispersion and the corresponding unit cell dimension are reported as a function of temperature. The error in the lattice parameter determination has also been calculated.

In the Myverol 18-99/P407 sample, the observed Bragg reflections can be ascribed to the coexistence of the above-mentioned cubic phases, the first being a bicontinuous cubic phase of spatial symmetry, Im3m, and the second belonging to the bicontinuous cubic Pn3m spatial symmetry. Note that the intensity of the Pn3m peaks was quite lower with respect to the intensities related to the Im3m phase (data not shown).

The increase in temperature up to 37°C resulted in the reduction of the lattice parameter, a, in both phases, mainly due to dehydration phenomena occurring at the lipid-water interface[33] (**Table 7**). Moreover, temperature was found to promote the formation of the Im3m bicontinuous cubic phase with respect to the Pn3m, as observed by Wörle et al.[29] In fact, at 37°C the reflections of the Im3m were more intense with respect to the peaks detected at 25°C. The diffraction pattern of the RYLO MG19/P407 sample at 25°C evidenced the presence of diffraction peaks corresponding to the Im3m phase and few peaks with low intensity corresponding to the Pn3m phase (data not shown). As for the Myverol 18-99-based sample, an increase in temperature to 37°C resulted in an increase in the Im3m reflections, while no differences in the intensity of the Pn3m peaks were observed. In the case of RYLO MG19/P407, a temperature increase of 12°C did not affect the lattice parameter, which remained constant after heating (**Table 7**).

To better characterize the spatial symmetry of the systems studied, further experiments with more powerful x-ray sources have been planned.

Stability Studies

The monoglyceride dispersions appear opalescent, whitish, and odorless. After production and elimination of large particles by filtration, dispersions were stored in glass vials at room temperature in the dark. In order to assess the physical stability of nanosome dispersions, organoleptic and morphological aspects such as phase separation and formation of precipitates were

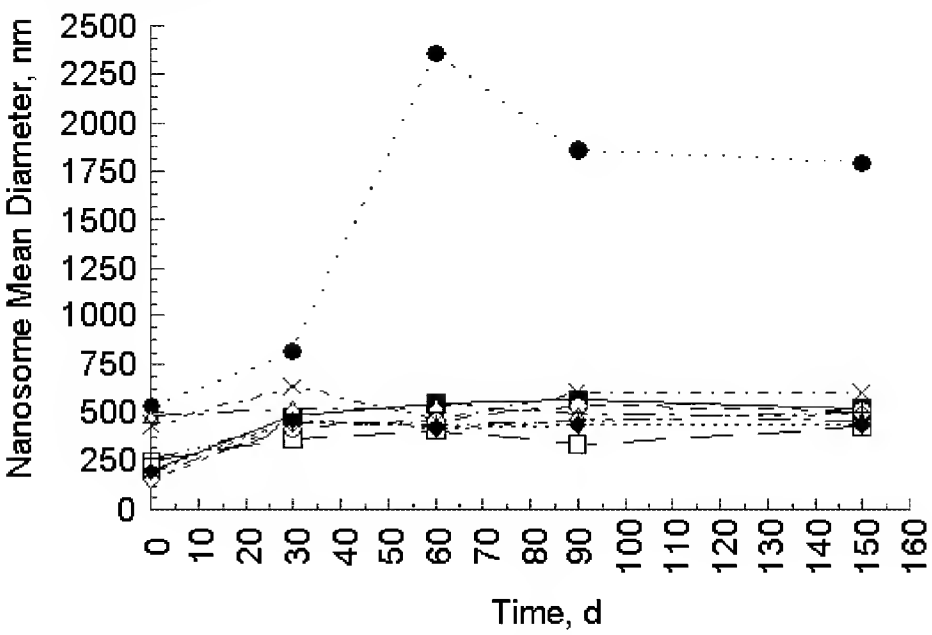

Figure 6. Variations of nanosome size by time. Determinations were performed on nanosome dispersions produced in different conditions. Dispersions were alternatively produced in the presence of poloxamer 0 (●), 2.5 (Δ), 5 (×), 7.5 (+), or 10 (♦) % wt/wt with respect to the disperse phase or in the presence of solutions of PVA 1 (□), 2.5 (○), 5 (◊) % wt/wt, finally dispersions were produced by monooleine (■) and the standard conditions described in the text. Mean diameters were determined by PCS and expressed as Intensity mean. Data represents the mean of 4 independent determinations. SD was always between ± 0.5%. Particle dimensions higher than 1 μm were confirmed by freeze-fracture electron microscopy observations

investigated as a function of time. It is noteworthy that the organoleptic and morphological aspects of nanosome dispersions do not change with time; nanosomes in fact are free from phase separation phenomena for almost 1 year from production.

Moreover PCS studies were conducted at different time intervals (from 0 to 5 months from production) in order to evidence possible variations in mean diameter of nanosomes over time.

Determinations were performed on dispersions produced in different experimental conditions, as a function of type of lipid phase, concentration of poloxamer, and presence of PVA in the external aqueous phase (**Figure 6**). As a general behavior, nanosomes undergo an increase in their dimensions within the first month after production. Nanosomes generally maintain their dimensions in the successive 4 months. Only in the case of nanosomes produced in the absence of poloxamer does mean diameter dramatically increase from 536 to 2357 nm after 2 months from production, afterwards the nanosomes stabilize to 1790 nm (after 5 months). Apart from these latest cases, nanosome diameters do not exceed 595 nm after 5 months from their production.

CONCLUSION

The emulsification of monoglyceride/surfactant mixtures in water results in the formation of aqueous dispersions composed of large lipid particles and nanosomes. The preformulatory study presented here enabled an assessment of the standard condition for the production of dispersions presenting 28% of larger irregular particles and nanosomes characterized by spheroidal shape, few aggregates, mean diameter of 193.5 nm, and high percentage of recovery (88% wt/wt).

Cryo-TEM analyses performed both on Myverol 18-99- and RYLO MG19-based dispersions demonstrated the coexistence of spherical vesicles and few faceted particles with well-shaped cubosomes. X-ray diffraction data revealed the coexistence of 2 different cubic

phases, the first being a bicontinuous cubic phase of spatial symmetry Im3m (Q^{229}) and the second belonging to the Pn3m (Q^{224}) spatial symmetry.

PCS studies showed the dimensional stability of nanosome dispersions that undergo an increase in mean diameter during the first month, followed by a stabilization in the subsequent 4 months from production.

Monoglyceride-based nanosome dispersions can be proposed for topical use, such as for percutaneous or mucosal applications. For instance, nanosome dispersions, because of the microbicidal properties of monoglycerides, could be designed for intravaginal treatment of sexually transmitted pathologies caused by viruses (eg, HSV, human immunodeficiency virus (HIV)) or by bacteria (eg, *Chlamydia trachomatis* and *Neisseria gonorrheae*).[4]

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